THE JOURNAL OF IMMUNOLOGY
Vol. 96, No


# IMMUNOLOGIC STUDIES OF ANTHRAX[1]

## IV. EVALUATION OF THE IMMUNOGENICITY OF THREE COMPONENTS OF ANTHRAX TOXIN

B. G. MAHLANDT, F. KLEIN, R. E. LINCOLN, B. W. HAINES, W. I. JONES, JR. AND R. H. FRIEDMAN[2]

THE JOURNAL OF IMMUNOLOGY
Vol. 96, No. 4

Printed in U.S.A.

# IMMUNOLOGIC STUDIES OF ANTHRAX[1]

## IV. EVALUATION OF THE IMMUNOGENICITY OF THREE COMPONENTS OF ANTHRAX TOXIN

B. G. MAHLANDT, F. KLEIN, R. E. LINCOLN, B. W. HAINES, W. I. JONES, JR. AND R. H. FRIEDMAN[2]

*From the United States Army Biological Laboratories, Fort Detrick, Frederick, Maryland*

Received for publication August 2, 1965

The antigenic toxin of *Bacillus anthracis* has been shown to be composed of three components identified as Factors I, II and III by English workers (1) and respectively as edema factor (EF), protective antigen (PA) and lethal factor (LF) by American workers (2). The development of "protective antigen" as an immunogen to *B. anthracis* was recently summarized by Lincoln *et al.* (3). They noted that certain of the anthrax toxin components were not used as immunogens in spite of the fact that certain strains of anthrax were found "refractive" to immunity by the "protective antigen" (4, 5). Recently, Stanley and Smith (1) reported on the degree of immunogenicity in guinea pigs of the three components and certain of their combinations. They concluded that Factor II immunized by itself. Factor I provided an additive effect but the immunogenicity of Factor I plus II was decreased by the addition of Factor III.

The pathophysiologic effects of the toxin components are somewhat better understood; their study shows that resistance to establishment of disease and susceptibility to its course are individual functions (6, 7). Immunity to anthrax, therefore, must be considered with respect to both factors, i.e., establishment as well as course of the disease. Of interest, too, is the development of basic information that may be used to develop an improved antiserum for use in treatment of anthrax.

In the work reported here, we have considered the possibility that immunity may differ in different hosts and have attempted to evaluate in the resistant rat and the susceptible guinea pig the independent functions of resistance to establishment of disease (immunity in the classical sense) and of change of resistance in the course of the disease after its establishment. This evaluation used animals immunized to all the factorial combinations of the three components of toxin with the antigen at three dose levels. Rats were challenged with sterile toxin and both the rat and guinea pig were challenged with virulent spores. Five criteria used to evaluate the efficacy of the immunogens were: (*1*) the serum antibody level as determined by the Ouchterlony technique, (*2*) the units of *in vitro* toxin neutralized by antibodies in the prechallenge serum, (*3*) the immunity index that quantitates the increase in dose of spores required to cause an immunized host to respond in the same length of time as the nonimmunized host, (*4*) the level of toxin in the blood at the time of death of the host challenged with spores and (*5*) the number of bacilli in the terminal blood of the host challenged with spores.

### MATERIALS AND METHODS

*Animals.* Male Fischer 344 rats, weighing 200 to 300 g, and Hartley guinea pigs from the Fort Detrick Animal Farm, weighing 300 to 400 g, were used.

*Challenge.* A $1 \times 10^9$ spore culture of the virulent Vollum V1b strain of *B. anthracis* was used to challenge the guinea pig, and spores ($1 \times 10^8$) plus egg-yolk medium, (Kaga, 8) were used to challenge rats. Whole anthrax toxin containing 30 units/ml, (Haines, Klein and Lincoln, 9) was used for the toxin challenge.

*Preparation of toxin and fractionation into components.* The method used for preparing anthrax toxin is described by Haines *et al.* (9). Horse serum was not added after centrifugation,

[1] In conducting the research reported herein the investigators have adhered to "Principles of Laboratory Animal Care" established by the National Society of Medical Research.

[2] Present address: 2634 San Antonio Drive, Walnut Creek, California 94598.

rather the LF, EF and PA components were separated by the method of Beall, Taylor and Thorne (2). The eluates containing EF were combined and passed through a diethylaminoethyl (DEAE) cellulose column to remove traces of LF. The components were dialyzed against distilled water at pH 7.5, dried under a vacuum of 10–30 $\mu$ of mercury for 21 hr in 1 ml quantities on an Amineo lyophilizer (American Instrument Co., Silver Spring) and stored at −25°C.

*Assay activity of components.* The agar diffusion method employed by Thorne and Belton (10) was used to quantitate the antigenic activity of each component. Using a 1:8 dilution in gelatin phosphate of the Fort Detrick horse antiserum (DH-1-10A), the minimum amount of LF detectable was 226 $\mu$g dry wt, equivalent to 25 $\mu$g protein. With this same antiserum the minimum amount of PA detectable was 137 $\mu$g dry wt, equivalent to 17 $\mu$g protein. EF could not be detected by this method. Protein was determined by the method of Waddel (11). When five times the minimal detectable amounts of PA and LF were placed in adjacent wells, single intersecting lines formed. The sensitivity of this technique, however, does not preclude the presence of other antigens in these preparations.

The biologic activity of EF was titrated by the method of Smith *et al.* (12). EF (combined with PA) was the only component that gave a positive reaction in guinea pigs. The minimum amount of EF detectable by this method was 57 $\mu$g dry wt, equivalent to 18 $\mu$g protein.

The biologic activity of LF was determined by intravenous (i.v.) injection in Fischer 344 rats of 2.0 ml of a mixture consisting of one volume of LF component plus four volumes of PA. This ratio constituted 33.6 lethal units/2 ml of toxin and killed rats in 104 min.

*Tests for immunogenicity.* All animals were injected intraperitoneally (i.p.) with 0.1-ml amounts of components using the protocol described by DeArmon *et al.* (13) and Klein *et al.* (14). Three levels of each component were administered to each of 32 rats and 16 guinea pigs. All animals and all component combinations were randomized.

The level of immunity attained was described in terms of the immunity index (13). This index represents the logarithmic difference in challenge dose required to cause the same time of response (death) in both immunized and control animals.

*Experimental procedures.* Two replications were performed for each type of challenge and for each host. The toxin-challenged rats provided three measures of response, (*1*) serum neutralizing potential, (*2*) serum antibody titer and (*3*) time to death. The spore-challenged animals, both rats and guinea pigs, provided, in addition to the above measurements, two more measurements, (*1*) concentration of bacilli/milliliter of blood at death and (*2*) units of toxin/milliliter of terminal blood.

One week following completion of the immunization protocol, 8 of each set of 32 rats and 12 of each set of 16 guinea pigs were bled by cardiac puncture. Approximately 1.2 ml of blood was withdrawn, centrifuged, and the serum decanted. One tenth of one milliliter of this prechallenge serum from each of the blood samples was combined with 30 units of crude anthrax *in vitro* toxin as defined by Haines *et al.* (9). Each of the combinations (toxin plus serum) was injected into one assay rat and the time to death was recorded. Prechallenge serum also was tested on Ouchterlony diffusion plates for antibody levels, using parallel holes of alignment.

## RESULTS

In the discussion of the data in this section all statistically significant treatments or interactions are mentioned.

*Resistance of immunized rats to toxin and to spore challenge.* Regardless of whether rats were immunized with any of the three components alone or in combination, only the LF component protected against challenge with toxin. Response ratios and harmonic mean response times of the rats that died are shown in Table I. The LF 100 $\mu$g treatment resulted in a high percentage of survivors. It was, therefore, not appropriate to calculate this statistic. However, the average time to death of those responding (dying) was extended 46 min, a 66% increase in response time. The 1000 $\mu$g concentration saved 127 of 128 immunized animals from death following toxin challenge regardless of PA or EF treatments. The component PA by itself appeared to have a small effect in prolonging the response time of rats to toxin challenge but apparently is synergistic with LF, since 34 of 43 rats protected with 1000 $\mu$g of PA plus 100 $\mu$g of LF survived toxin challenge. The component EF gave essentially no protection. Thus, it was apparent

TABLE I

*Response ratios and harmonic mean response times of immunized rats challenged with whole toxin*

| Immunization Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|
| EF[a] | LF[a] | PA[a] | | | | | |
| | | 0 μg | | 100 μg | | 1000 μg | |
| | | Ratio[b] | MTD[a] | Ratio | MTD | Ratio | MTD |
| μg | μg | | min | | min | | min |
| 0 | 0 | 16/16 | 70 | 15/15 | 73 | 15/15 | 85 |
| 0 | 100 | 15/15 | 78 | 14/14 | 73 | 4/12 | —[c] |
| 0 | 1000 | 0/15 | — | 0/14 | — | 0/15 | — |
| 100 | 0 | 14/14 | 74 | 15/15 | 69 | 8/15 | — |
| 100 | 100 | 13/13 | 73 | 14/15 | 82 | 2/16 | — |
| 100 | 1000 | 0/12 | — | 1/15 | — | 0/15 | — |
| 1000 | 0 | 12/12 | 73 | 15/15 | 72 | 9/14 | — |
| 1000 | 100 | 11/13 | 109 | 15/15 | 75 | 3/15 | — |
| 1000 | 1000 | 0/13 | — | 0/14 | — | 0/15 | — |

[a] EF = edema factor; LF = lethal factor; PA = protective antigen; MTD = mean time to death.
[b] Number dead/number challenged.
[c] No mean response times were calculated when 50% or more of the animals survived.

TABLE II

*Harmonic mean response times and immunity indexes of immunized rats challenged with virulent spores*

| Immunization Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|
| EF[a] | LF[a] | PA[a] | | | | | |
| | | 0 μg | | 100 μg | | 1000 μg | |
| | | MTD[a] | I[a] | MTD | I | MTD | I |
| μg | μg | hr | | hr | | hr | |
| 0 | 0 | 16.4 | 0 | 14.9 | −0.8 | 13.2 | −1.8 |
| 0 | 100 | 14.8 | −0.8 | 19.7 | 1.3 | 25.0 | 2.7 |
| 0 | 1000 | 19.5 | 1.2 | 21.1 | 1.7 | 16.0 | −0.2 |
| 100 | 0 | 14.1 | −1.2 | 14.6 | −0.9 | 16.6 | 0.1 |
| 100 | 100 | 17.6 | 0.5 | 20.8 | 1.7 | 23.3 | 2.3 |
| 100 | 1000 | 23.3 | 2.3 | 17.2 | 0.4 | 24.1 | 2.5 |
| 1000 | 0 | 13.2 | −1.9 | 18.5 | 0.9 | 28.5 | 3.3 |
| 1000 | 100 | 17.6 | 0.5 | 16.5 | 0.1 | 25.7 | 2.8 |
| 1000 | 1000 | 33.5 | 4.0 | 27.1 | 3.1 | 23.1 | 2.3 |

[a] EF = edema factor; LF = lethal factor; PA = protective antigen; MTD = mean time to death; I = immunity index.

that the LF component was highly immunogenic in rats against challenge with anthrax toxin.

The analysis of variance for rats immunized similarly to those above but challenged by spores (Table II) indicates that only LF had a highly significant effect, while EF interacted with LF significantly ($P < 0.03$) to increase the immunity index. Of 60 surviving animals, 46 were immunized with LF. PA interacted antagonistically with LF ($P < 0.05$).

*Immunogenicity for guinea pigs of the three toxin components against spore challenge.* The immunity indexes of the three components singly or in combination were calculated from the response time and are shown in Table III. It is significant to note that again the LF component gave strong protection against the spore challenge, as did PA. Analysis indicates that the response to both of these components was statistically significant ($P < 0.01$).

The marked interaction ($P < 0.01$) between the LF and PA components is summarized in

TABLE III

*Harmonic mean response times and immunity indexes of immunized guinea pigs challenged with virulent spores*

| Immunization Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|
| EF[a] | LF[a] | PA[a] | | | | | |
| | | 0 μg | | 100 μg | | 1000 μg | |
| | | MTD[a] | I[a] | MTD | I | MTD | I |
| μg | μg | hr | | hr | | hr | |
| 0 | 0 | 20.3 | 0 | 25.4 | −0.3 | 47.3 | 2.3 |
| 0 | 100 | 24.8 | −0.5 | 29.9 | 1.1 | 39.6 | 3.0 |
| 0 | 1000 | 36.1 | 2.5 | 40.9 | 3.2 | 35.7 | 2.4 |
| 100 | 0 | 26.4 | 0.0 | 31.3 | 1.5 | 40.0 | 3.1 |
| 100 | 100 | 25.0 | −0.5 | 27.7 | 0.5 | 40.2 | 3.1 |
| 100 | 1000 | 41.9 | 3.4 | 39.5 | 3.0 | 44.2 | 3.7 |
| 1000 | 0 | 22.5 | −1.5 | 31.8 | 1.6 | 50.5 | 4.3 |
| 1000 | 100 | 25.5 | −0.3 | 29.2 | 0.9 | 45.2 | 3.8 |
| 1000 | 1000 | 31.6 | 1.5 | 35.0 | 2.3 | 46.8 | 4.0 |

[a] EF = edema factor; LF = lethal factor; PA = protective antigen; MTD = mean time to death; I = immunity index.

Table IV. PA and LF each had the strongest effect in the absence of the other. When 100 μg of either PA or LF was present, 100 μg of the other adds no beneficial effect, but 1000 μg of the other does extend the response time. The EF component neither had an immunogenic effect alone nor did it have an antagonistic effect on PA or LF. The data show that this interactive effect was opposite to that observed in rats.

*Number of organisms and units of toxin/milliliter of terminal blood of guinea pigs and rats as affected by immunization.* Terminal blood of rats and guinea pigs was assayed for number of viable organisms and concentration of toxin. The interaction among the terminal variables in the susceptible guinea pig and the resistant rat are graphically illustrated in Figure 1. In general, it appears that immunization extends the time to death, as evidenced by the Immunity Index, I, of both the susceptible and resistant animals. It also was apparent that as resistance attributable to immunity increases, the terminal number of organisms and units of toxin/milliliter of blood decreases in the guinea pig, but little or no influence on these parameters could be detected in the rat.

In the guinea pig both PA and LF affected the number of bacilli/milliliter of blood at death ($P < 0.01$). These same treatments as well as the PA × LF ($P < 0.05$) and PA × EF ($P < 0.05$) and EF × PA × LF ($P < 0.01$) interactions affected the number of bacilli/milliliter of blood,

TABLE IV

*Summary of interactions shown in Table III between PA[a] and LF[a, b]*

| Immunization Treatments | | | |
|---|---|---|---|
| LF | PA | | |
| | 0 μg | 100 μg | 1000 μg |
| μg | | | |
| 0 | 24.8 | 29.2 | 45.7 |
| 100 | 25.1 | 28.9 | 41.5 |
| 1000 | 26.1 | 38.3 | 41.8 |

[a] PA = protective antigen; LF = lethal factor.
[b] EF (edema factor) had no effect so does not appear in this tabulation.

while the PA × EF and PA × LF interaction were significant ($P < 0.05$). None of the treatments had an influence on the toxin concentration of the terminal blood of rats.

EF (100 μg) increased the terminal concentration of organisms and of toxin ($11.4 \times 10^9$ organisms and 106 units/ml), but the number of bacilli/milliliter and of toxin of terminal blood returned nearly to control level ($8.8 \times 10^8$ organisms and 88 units/ml) when 1000 μg of EF was used. Although this effect was not statistically significant, the same pattern was observed in both hosts.

*Lack of demonstrable immunogenicity as measured by the Ouchterlony technique and serum-neutralization tests.* Prechallenge sera from animals of both species were assayed for antibody

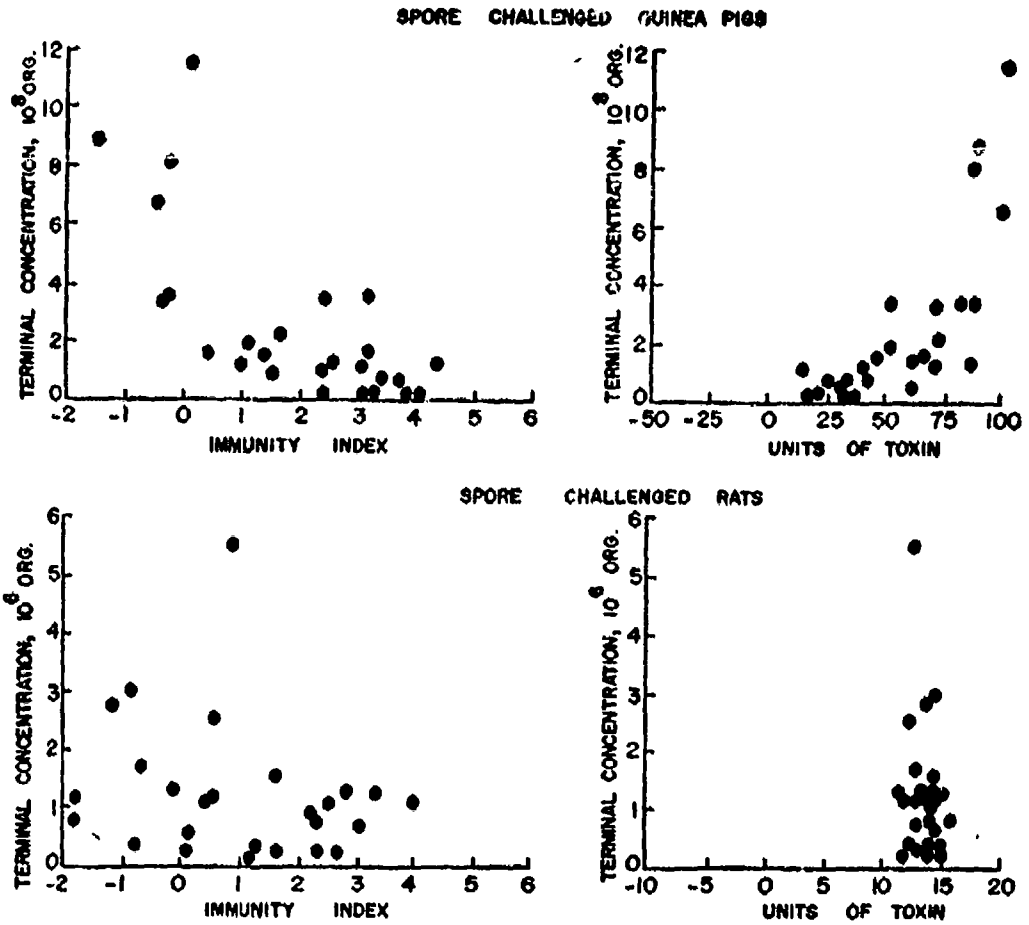


*Figure 1.* The interaction among the terminal variables in the immunized guinea pig and rat. Each data point represents one immunization protocol and is the mean of 9 to 10 animals distributed among two populations.

on Ouchterlony diffusion plates. The sera from rats gave uniformly negative results. Sera of 47 of the 270 prechallenged guinea pigs showed a titer. The distribution of the positive-reacting sera, based on immunization protocol, is given in Table V. PA had little effect in producing precipitin lines that were demonstrable on Ouchterlony plates, but LF was much more consistently successful in producing identifiable lines against the antiserum used in these studies. All titers were low, ranging from 1:4 to 1:8.

The neutralization tests of anthrax toxin by prechallenged blood serum for the rat and guinea pig revealed no significant toxin-neutralizing ability of any of the components or their combinations on the prechallenge sera from either species. This finding, however, does not preclude the possibility of a level of antibody below that detectable by the Ouchterlony test.

## DISCUSSION

In this study we found that LF is the only component that is highly immunogenic against spore challenge of both hosts, the rat and the guinea pig, and toxin challenge of the rat. In the rat, addition of PA to LF increased the resistance significantly against the spore challenge but added nothing to the efficacy of the immunization against toxin challenge. In contrast, the guinea pig was effectively protected against

TABLE V

*Distribution of prechallenge sera of 47 guinea pigs that showed titers of one fourth or greater following PA and LF immunization*[a, b]

| Immunization Treatments | | | |
|---|---|---|---|
| LF[a] | PA[a] | | |
| | 0 μg | 100 μg | 1000 μg |
| μg | | | |
| 0 | 0 | 4 | 3 |
| 100 | 2 | 0 | 4 |
| 1000 | 11 | 13 | 10 |

[a] LF = lethal factor; PA = protective antigen.
[b] EF (edema factor) did not have a significant effect so does not appear in this table.

spore challenge with either the LF or PA component and these components complemented each other.

This work raises the question of what model may be used to extrapolate from experimental animals to man. We find the rat responds quite differently to these immunizing antigens than does the guinea pig. It seems that the conservative view on the development of an antigen for human beings or domestic animals is to use the complete toxin, for there is no experimental basis for selection or use of a single antigen for immunizing man, domestic, or experimental animals. Indeed all evidence is contrary to this practice for the "protective antigen" of Gladstone (15) as well as later workers who used serum in the production or processing of the antigen was probably composed of all components of toxin.

These data also indicate the need for use of relatively large amounts of antigen, for even the use of 1000 μg of each antigen resulted in a relatively small proportion of animals developing a demonstrable level of antibody and was much lower than that obtained when a live vaccine was used in conjunction with complete toxin antigen (14). Considering the very low titers as well as partial response reported for man (16, 17), we would predict the need to use larger amounts of improved antigen.

The work of Stanley and Smith (1) and this report differ in that we reported a lack of effect of EF on resistance in our tests, whereas the British workers report a positive effect. These differences may lie in the undetected contamination of one component with another in its active or toxoid form. In any case some discrepancy between our results and those of Stanley and Smith (1) are not unexpected because they (*a*) generated information only on the guinea pig in much more limited experimentation, and (*b*) used only one criterion, i.e., challenge with 1000 lethal doses of spores, to evaluate immunogenicity of the antigens. The present authors definitely question the repeated statement by Smith (1, 18) that the optimum proportions of the antigens cannot be determined because of the large number of animals required. Determination of the optimum proportions and concentration of antigens is a matter of desire and planning, since quantitative methods such as the immunity index (13) and modern statistical designs, combined with evaluation of multiple criteria of immunity as used in this work, allow the efficient acquisition of the necessary data.

*Acknowledgments.* We are grateful to Mr. Charles C. Wigington, Mr. Byron U. Ross, SP5 James P. Dobbs, SP5 Kenneth J. Hendrix and Mrs. Shirley E. Snowden for their technical assistance in the completion of this work.

## SUMMARY

Three components of anthrax toxin (edema factor, protective antigen and lethal factor) were separated and tested singly at three dose levels and in factorial combination (27 treatments) to determine their efficacy as immunogens in a resistant host (rat) and in a susceptible host (guinea pig). Each treatment was evaluated as an immunogen by five criteria: (*1*) protection of the host against challenge, (*2*) influence on the number of bacilli/milliliter of blood at death, (*3*) change in the units of toxin/milliliter of blood at death, (*4*) a development of antibody titer (Ouchterlony) and (*5*) units of toxin neutralized/milliliter of blood.

These evaluations showed that (*1*) the LF component was highly immunogenic in rats against either toxin or spore challenge and in guinea pigs against spore challenge; (*2*) the PA component was immunogenic against spore challenge in rats and guinea pigs, but completely ineffective against toxin challenge in rats; (*3*) the EF component alone was nonimmunogenic; (*4*) the effects of LF and PA were additive and (*5*) EF added to LF, PA or LF-PA combinations interacted significantly with LF to increase re-

sistance in the rat, but was not additive in resistance in the guinea pig.

The number of organisms/milliliter of terminal blood decreased as resistance to establishment of disease increased. The units of toxin/milliliter of terminal blood were closely related to the number of bacilli/milliliter of blood at death

Only 17% of the prechallenge serum of guinea pigs, principally among the LF treatments, produced antigen antibody precipitin lines on Ouchterlony plates. The rat sera were all negative in this test.

The antigen used to immunize man and animals should contain all the toxin components for maximum efficiency.

## REFERENCES


1. Stanley, J. L. and Smith, H., J. Gen. Microbiol., *31:* 329, 1963.
2. Beall, F. A., Taylor, M. J. and Thorne, C. B., J. Bact., *83:* 1274, 1962.
3. Lincoln, R. E., Walker, J. S., Klein, F. and Haines, B. W., Advances Vet. Sci., *9:* 327, 1964.
4. Auerbach, L. and Wright, G. G., J. Immun., *76:* 129, 1955.
5. Ward, M. K., McGann, V. G., Hoggie, A. L., Huff, M. L., Knode, R. G., Jr. and Roberts, E. D., J. Infect. Dis., *115:* 59, 1965.
6. Klein, F., Haines, B. W., Mahlandt, B. G., DeArmon, I. A., Jr. and Lincoln, R. E., J. Bact., *85:* 1032, 1963.
7. Klein, F., Walker, J. S., Fitzpatrick, D. F., Lincoln, R. E., Mahlandt, B. G., Jones, W. I., Jr. and Hendrix, K. J., J. Infect. Dis., In press.
8. Kaga, M., Jap. J. Bact. *11:* 477, 1956.
9. Haines, B. W., Klein, F. and Lincoln, R. E., J. Bact., *89:* 74, 1965.
10. Thorne, C. B. and Belton, F. C., J. Gen. Microbiol., *17:* 505, 1957.
11. Waddel, W. J., J. Lab. Clin. Med., *48:* 311, 1956.
12. Smith, H., Tempest, D. W., Stanley, J. L., Harris-Smith, P. W. and Gallop, R. C., Brit. J. Exp. Path., *37:* 203, 1956.
13. DeArmon, I. A., Jr., Klein, F., Lincoln, R. E., Mahlandt, B. G. and Fornelius, A. L., J. Immun., *87:* 233, 1960.
14. Klein, F., Mahlandt, B. G., Lincoln, R. E., DeArmon, I. A., Jr. and Fornelius, A. L., J. Immun., *88:* 15, 1962.
15. Gladstone, G. P., Brit. J. Exp. Path., *27:* 394, 1946.
16. Norman, P. S., Ray, J. G., Brachman, P. S., Plotkin, S. A. and Pagano, J. S., Amer. J. Hyg., *78:* 32, 1962.
17. Brachman, P. S., Gold, H., Plotkin, S. A., Fekety, F. R., Herrin, M. and Ingraham, N. R., Amer. J. Public Health, *53:* 632, 1962.
18. Smith, H., 14th Symposium, Soc. Gen. Microbiol., Cambridge University Press, London, 1964.